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Original Article

First record of the crown rot fungus *Fusarium equiseti* affecting *Triticum aestivum* L. and *Aptenia cordifolia* in Iraq

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Abstract

During growing seasons of 2015 and 2016, severe rot symptoms were noticed epidemiologically on root and crown of *Triticum aestivum* and *Aptenia cordifolia* seedlings in Kerbala Province, Iraq. Thus, the aims of this research were to isolate, identify and assess pathogenicity of the causal agent of the disease. Several fungal isolates of *Fusarium* genus were consistently isolated from the diseased root and crown of the seedlings. The pathogen was identified as *Fusarium equiseti* based on its cultural and morphological characteristics, pathogenicity and sequence of the ITS-rDNA region. This is first record of crown rot disease caused by *F. equiseti* on *T. aestivum* and *A. cordifolia* plants in Iraq.

Keywords: Crown rot disease, *Fusarium equiseti*, Morphological and molecular identification, Iraq

Introduction

Wheat (*Triticum aestivum*), which belongs to Poaceae family, is considered one of the most economically essential food for approximately more than 1/3 of the global population (Bockus et al., 2010). It has been ranked as the fourth most important food source in the world, with global production more than 700 million tons (FAO, 2015). Wheat has multiple beneficial characteristics and properties such as its agronomic adaptation, simplicity of grain storage and easy processing into flour used to produce numerous edible and healthy food products (Orth and Shellenberger, 1988). It is also one of the main diet components due to its high content of carbohydrate, easily digested proteins, a variety of different lipids, fibers, minerals and vitamins (Khalil and Shrewry, 2009). Additionally, the low quality of wheat grains are a good source for animal fodder and produce some industrial products such as alcohol and paper additives (Zarrin et al., 2009; Bockus et al., 2010).

Heartleaf ice plant (*Aptenia cordifolia*) is from the family Aizoaceae. It is also called baby sun rose and red aptenia that is native to South Africa (Court, 2000). It is a perennial evergreen succulent plant with thick roots and rounded or angled stems, which grow in horizontal clusters on the soil (Sajeva and Costanzo, 2000). The leaves are glossy green in heart or oval shape-like structures arranging singly or in pairs. The flowers are red to magenta, emerging singularly or in clusters on the leaf axils and open throughout the day. They are self-fertilized leading to produce fruits in a capsule shape with multi-chambers containing brown to black seeds (Rowley, 1980; Smith and Vanwyk, 2008). It is widely used as an anti-inflammatory and an ornamental plant cultivated in different regions of Iraq (Vanwyk et al., 1997; Smith, 2005).

Crown rot disease is one of the common diseases that appears on different plant hosts causing a significant

reduction in yield and quality (Lazreg et al., 2013; Gebremariam et al., 2017). Diseased seedlings of the host plants show light brown necrotic lesions covering the roots and extending to crown regions, which latterly develop to be darker and larger. Subsequently, the diseased seedlings collapse and die (Paulitz et al., 2002; Moya-Elizondoand Jacobsen, 2016). Although this disease has not been found to be recorded on heartleaf ice plants (*A. cordifolia*) worldwide, it was reported on wheat plants (*T. aestivum*) in different countries including the USA (Gonzalez and Trevathan, 2000), Norway (Kosiak et al., 2003), Iran (Hajieghrari, 2009), Tunisia (Fakhfakh et al., 2011), Korea (Kim et al., 2016), Turkey (Gebremariam et al., 2017). Additionally, in Iraq, this disease was reported on wheat caused by *Fusarium avenaceum*, *F. solani*, *Rhizoctonia solani* and *Stemphylium* sp. (Salih et al., 2009) and *F. culmorum* (Khalifah and Matny, 2013). In fact, diverse pathogenic species of *Fusarium* genus including *F. equiseti* were reported to cause this disease (Rossi et al., 1995; Gonzalez and Trevathan, 2000; Gebremariam et al., 2017). However, so far in Iraq *F. equiseti* has not been identified neither on wheat *T. aestivum* nor on heartleaf ice *A. cordifolia* plants. Therefore, the purpose of this work is to investigate possibility of association the species *F. equiseti* with the crown rot disease on wheat and heartleaf plants in Iraq.

Material and Methods

Samples collection and pathogen isolation

To isolate the pathogen, seedling samples of *T. aestivum* and *A. cordifolia* showing symptoms of the crown rot disease were collected during a survey undertaken from December 2015 to April 2016 from several fields and plantations located in different regions of Kerbala Province/Iraq. The symptoms appeared on seedlings were brownish to black necrotic lesions accompanied with softness covering the roots and the basal stems. Later, the seedlings wilted, collapsed, rotted and died. The collected seedlings were transported to plant disease laboratory in Agriculture college/University of Kerbala and stored in fridge at 4 °C until further processing(Gebremariam et al., 2017; Hwang et al.,2017).

Symptomatic tissues from roots and basal stems of diseased seedlings were cut into 1-2 cm long pieces after washed gently with tap water and surface-sterilized in sodium hypochlorite (NaOCl) 1 % (w/v). These symptomatic tissues were then dried using sterilized filter paper and placed on water agar (WA) plates, followed by incubation at 25°C in darkness. Hyphal tip from each emerging colony was cut and transferred into potato dextrose agar (PDA) plates containing amoxicillin (200 µg/ml). Subsequently, all plates were incubated at 25 °C under darkness for 5-6 days (Gebremariam et al., 2017; Maridueña-Zavala et al., 2016; Hwang et al., 2017).

Morphological identification

To determine the morphological characteristics including mycelial structure, colour and growth ratio of all pure fungal cultures obtained were monitored and measured after sub-culturing them on synthetic low-nutrient agar (SNA).The conidia and chlamydospores of the pure isolates were also observed after 7 and 14 days using a compound microscope (HumaScopePremiumLED, Germany) that was equipped with an ocular micrometer measurement. Subsequently, shapes and measurements of the fungal conidia and chlamydospores were compared with former descriptions (Burgess et al., 1994; Leslie and Summerell, 2006).

Pathogenicity

To assess pathogenicity of the *Fusarium* sp. isolates, healthy seedlings at two-true leaf stage of *T. aestivum* and *A. cordifolia* grown on water agar media were transplanted into autoclaved and inoculated compost with the isolates of *Fusarium* sp. The inoculated compost was made by mixing spore suspension of a pure 7 days isolate culture in one plate with 0.5 kg of autoclaved commercial compost. The mixture was incubated for three days at 25 °C in darkness with shaking every day. The mixture was then distributed equally into surface-sterilized plastic pots. In each full pot, three seedlings of each plant were transplanted. In addition, healthy plants of each host were planted in sterile compost as a control. Subsequently, all pots were placed in a growth cabinet under conditions 16 h lightat 25°C and 8 h dark at 15°C with 70% humidity (Mitter et al., 2006). Watering of seedlings was performed as needed and monitored daily until symptoms of crown rot disease were observed. Re-isolation of *Fusarium* sp. was made from symptomatic seedlings of both plants as previously described. The morphological characteristics of the re-isolated *Fusarium* sp. isolates were compared with the corresponding *Fusarium* isolates to accomplish the

requirement of Koch`s postulates. No *Fusarium* sp. infection was seen in all control plants of both hosts.

Molecular identification

The total genomic DNA from pure *Fusarium* isolates was extracted using the DNeasy Plant Mini kit (Qiagen, Germany) following the manufacturer`s instructions. Polymerase chain reaction (PCR) test was applied using the universal primer set (ITS1/ITS4) to amplify the internal transcribed spacer (ITS) region of fungi (White et al., 1990). The PCR amplicons were sequenced at Macrogen in Seoul, South Korea (ABI 3730xI automated Sequencer; Applied Biosystems). Analysis of chromatograms and nucleotide sequences of sequencing resulting were conducted using Chromas software version2.6.4. Then, Basic Local Alignment Search Tool (BLAST) was operated for comparison the obtained sequences with sequences database of the GenBank at the National Center for Biotechnology Information National (NCBI). Another comparison was operated with database of the Fusarium MLST website for same purpose. Subsequently, the Molecular Evolutionary Genetics Analysis (MEGA) version6.06 was applied for phylogenetic analysis sequence data and operating the Neighbour joining method of the ITS-rDNA *Fusarium* sp. sequences of this study and others recorded in the NCBI to construct a phylogenetic tree (Tamura et al., 2011). Edited sequences of Fusarium isolates were deposited to NCBI-GenBank Database for recording.

Results and Discussion

Isolation and morphological identification

A fungus was consistently isolated from almost all symptomatic seedlings of *T. aestivum* and *A. cordifolia*. The colony growth of this fungus on SNA was rapid, reached more than 5 cm diameter after 6-7 days. Initially, the appearance of colonies was white aerial mycelia that later became brown and bright orange representing the causal agent – sporodochia (Figure 1 A-B). The reverse surface of the colony was bronze to brown, with dark brown spots. After 2 weeks incubation at 25 °C in darkness, an enormous collection of colorless conidia and chlamydospores with various sizes and shapes were observed and measured. Although the microconidia were not seen, the macroconidia were distinctly curved and falcate with thick-wall and the majority had 3-5 septate. Their apical cells were acute and showed an extended enhanced curving of the macroconidia while the basal cells were distinctly foot shape. Conidial sizes were extremely variable with average length 20-70 µm and width 2.5-6.5 µm (Figure 1 C-D). Additionally, the chlamydospores were spherical, 6.8–9 µm in diameter, either as chains or clusters with very obvious thick walls. The conidiophores were noticed to be either unbranched or loosely branched mono to tri-phialides. Depending on the morphological appearances of the fungal isolates, they were initially identified as *Fusarium equiseti* (Corda) Sacc. (Leslie and Summerell, 2006; Burgess et al., 1994).

Molecular identification

The PCR amplicons size of the ITS-rDNA region amplified were more than 600 bp in both isolates. The sequences were recorded at NCBI-GenBank with accession numbers MF099868.1 of *T. aestivum* and MF099783.1 of *A. cordifolia*. BLAST analysis of ITS-rDNA sequences of the two isolates showed that the first sequence shared 99% similarity to the known sequences of *F. equiseti* deposited in GeneBank database with accession numbers KX343174.1, KR094440.1 and KY318493.1. However, the second sequence demonstrated 100% identity to those sequences of published *F. equiseti* isolates with accession numbers KY560313.1 and EF483926.1. The phylogenetic analysis revealed that the two isolates respectively grouped with several reference isolates of the fungus *F. equiseti* (Figures 2 and 3). Thus, based on these results the fungus was confirmed to be *F. equiseti*.

Pathogenicity

The crown rot disease incidence of the inoculated *T. aestivum* and *A. cordifolia* seedlings was approximately 100% whereas the non-inoculated plants of both hosts did not reveal any of the disease symptoms. After 3 weeks, the seedlings showed symptoms similar to those noticed on diseased seedlings in the infested fields and plantations. These symptoms included wilting, brown lesions of the basal stems and roots that eventually progressed to rotting and collapsing of the seedlings. It was also observed that the height of the inoculated seedlings was notably reduced compared with the control seedlings (Figure 1E-F).The causal agent, *F. equiseti*, was re-isolated from all inoculated seedlings of both hosts and its microscopic features were similar to the original isolates. Consequently, Koch`s postulates were achieved successfully and revealed that *F. equiseti*

was the causative agent of the crown rot disease on both plant hosts.

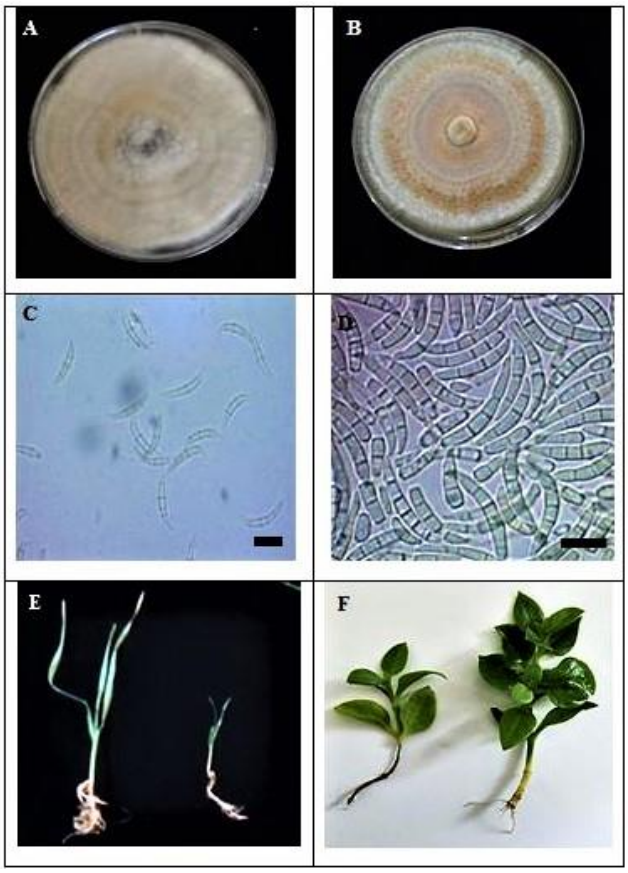

Figure 1: (Colour online) Cultural, morphological characteristics of *F.equiseti* and crown rot symptoms on *T. aestivum* and *A. cordifolia* seedlings. Colony of *F. equiseti* grown on PDA after 7 days (A) and 14 days (B). Macroconidia after 7 days (C) and 14 days (D). Crown rot symptoms appear on inoculated *T. aestivum* (E) the left plant is control while the right plant is diseased, on inoculated *A. cordifolia* plant (F) the left plant is diseased whereas the right is control. Note, in diseased plants roots and base stems (crown) are brown and rotted.
Bars: C–D = 20 µm.

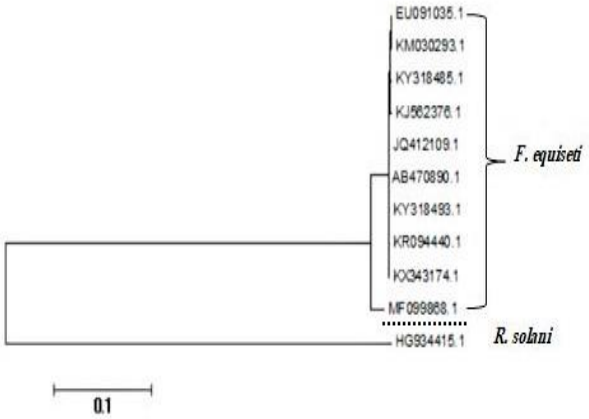

Figure 2: Phylogenetic tree created with the ITS-rDNA sequences of the *Fusarium equiseti* isolated from *T. aestivum* seedlings in this study (determined by dashed line) and other isolates of the same species retrieved from GenBank. Bootstrap values were based on 1000 replicates. The out-group species used was *Rhizoctonia solani*.

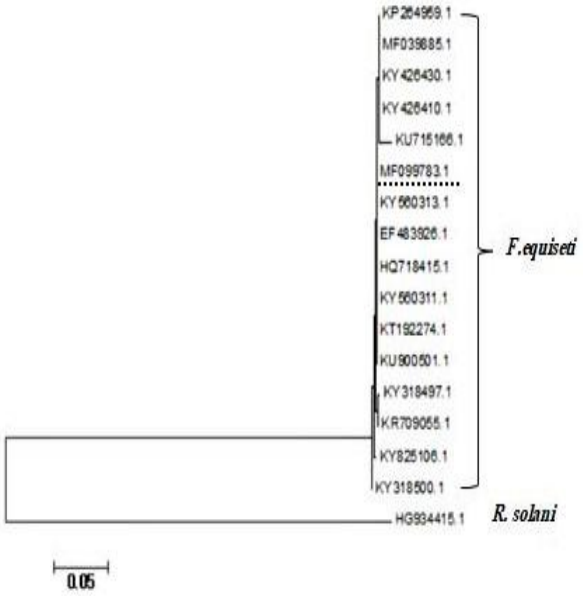

Figure 3: Phylogenetic tree constructed using the ITS-rDNA sequences of the *F. equiseti* obtained from the diseased *A. cordifolia* seedlings in this study (determined by dashed line) and other isolates of the same species retrieved from GenBank. Bootstrap values were depended on 1000 replicates. *R. solani* was the out-group species.

The current study has shown that *F. equiseti* is the causative agent of the crown rot disease on *T. aestivum* and *A. cordifolia* seedlings in Kerbala Province, Iraq. Identification of this pathogen was based on its morphological and molecular characteristics in addition to pathogenicity ability. The morphological features of the macroconidia, chlamydospores and conidiogenesis of both *F. equiseti* isolates were compatible with previous studies descriptions (Burgess et al., 1994; Leslie and Summerell, 2006; Li et al., 2017). It was observed clearly that the macroconidia were in diverse sizes and distinctively curved with more than 3 septate. Their apical cells were fusiform whereas the basal cells were in foot shape like-structure. Furthermore, the chlamydospores were globule shaped carried either in chains or clusters. However, the microconidia were not observed. Additionally, amplification of the ITS region yielded amplicons exceeding 600bp for both isolates and the phylogenetic analysis based on the ITS sequences demonstrated that both representative isolates grouped within a clade that included several known isolates of *F. equiseti* (Figures 2 and 3).

In the pathogenicity test, the *F. equiseti* was able to re-infect of *T. aestivum* and *A. cordifolia* seedlings producing the typical symptoms of crown rot that were similar to those observed in the infested fields and plantations previously.

Numerous studies were reported *F. equiseti* infecting *T. aestivum* and other plants of Poaceae family such as *Hordeum vulgare*, *Avena sativa* (Goswami et al., 2008) and *Zea mays* (Li et al., 2014). Furthermore, this pathogen was found to infect numerous members of Fabaceae family such as *Pisum sativum*, *Phaseolus vulgaris*, *Medicago sativa* and *Cicer arietinum* (Goswami et al., 2008). As well as, it infects *Vigna unguiculata* (Rodrigues and Menezes, 2005). Additionally, several other studies were revealed that *F. equiseti* infects other host plants belonging to different families for instance *Pinus halepensis* (Lazreg et al., 2013), *Cuminum cyminum* (Ramch and raand Bhatt, 2012), *Carya illinoinensis* (Lazarotto et al., 2014) and *Brassica oleracea* var. *botrytis* (Li et al., 2017). Although, *F. equiseti* is a plant pathogen prevalent worldwide infecting a wide range of plant hosts, it has not been reported in Iraq infecting *T. aestivum* and *A. cordifolia* plants. To the best of our knowledge, *F. equiseti* causing crown rot on *T. aestivum* and *A. cordifolia* plants is first report in Iraq.

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